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Plasmodium-associated changes in human odor attract mosquitoes

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Abstract

Malaria parasites (*Plasmodium*) can change the attractiveness of their vertebrate hosts to *Anopheles* vectors, leading to a greater number of vector-host contacts and increased transmission. Indeed, naturally *Plasmodium*-infected children have been shown to attract more mosquitoes than parasite-free children. Here, we demonstrate *Plasmodium*-induced increases in the attractiveness of skin odor in Kenyan children, and reveal quantitative differences in the production of specific odor components in infected, versus parasite-free, individuals. We found the aldehydes heptanal, octanal and nonanal to be produced in greater amounts by infected individuals, and detected by mosquito antennae. In behavioral experiments, we demonstrated that these, and other, *Plasmodium*-induced aldehydes enhanced the attractiveness of a synthetic odor blend mimicking 'healthy' human odor. Heptanal alone increased the attractiveness of 'parasite-free' natural human odor. Should the increased production of these aldehydes by *Plasmodium*-infected humans lead to increased mosquito biting in a natural setting, this would likely affect the transmission of malaria.

Significance Statement

In vector-borne disease systems, there is mounting evidence that vertebrate hosts become more attractive to disease vectors during infection, yet in human malaria, the underlying mechanism has not been studied. We identified compounds, including aldehydes, that are produced in relatively greater amounts in the skin odor of individuals with malaria, thus, demonstrating a basis for this phenomenon in the cues used during mosquito host location. By establishing the attractiveness of these compounds to malaria mosquito vectors in laboratory bioassays, we characterise a process by which *Plasmodium* infection of humans could lead to increased mosquito biting. These compounds may serve as biomarkers of malaria, or be used to enhance the efficacy of chemical lures used to trap mosquitoes.

Introduction

Parasite transmission often constitutes a population bottleneck: of the many parasites within one host, only a few are successfully transmitted to the next (1). Hence, parasites often evolve to exert influence over the transmission events. The malaria parasite *Plasmodium* would benefit from increasing its infected vertebrate host's attractiveness to susceptible *Anopheles* mosquito vectors, if this resulted in increased contact rates between the two hosts. Such changes in attractiveness have previously been demonstrated in both animal (2–6) and human (7–9) malaria systems, as well as in other vector-borne disease systems (10–13). While manipulation of the 'attractiveness' phenotype by the parasite has been suggested (5–9), it is difficult to disentangle this from some by-product of infection that fortuitously leads to increased host attractiveness and subsequently transmission. Body odor, comprising the volatile compounds emitted from the skin of vertebrates, is the most important cue used by *Anopheles* for host location (14). It has been shown that differences in the composition of skin odor are responsible for the variation in attractiveness to biting insects known to exist between people (15, 16), and these differences may be influenced by body weight and/or surface area, hormones or genetic factors (17–19). Human body odor can also be influenced by disease, including metabolic disorders, genetic disorders, and infections (20). A study of *Plasmodium* infection in mice found such changes in body odor to be associated with changes in attractiveness to mosquitoes (6), and another found compositional changes in skin odor during controlled human malaria infection (CHMI), with a variable effect on attractiveness (21). While increased attractiveness of *Plasmodium*-infected individuals has been demonstrated in a malaria-endemic setting (9), remarkably, no study has yet investigated the skin chemistry underlying this phenomenon. Given the crucial importance of body odor to mosquito host location, and the proposition that body odor can be altered during disease, here, we hypothesize that infection with *Plasmodium* parasites changes the odor of humans, and that this influences attractiveness of humans to mosquitoes. To test this hypothesis, we first confirmed that asymptomatic children in Western Kenya were more attractive to mosquitoes when harbouring *Plasmodium* parasites, before comparing skin odor composition between *Plasmodium*-infected and parasite-free children from the same population. Using analytical chemistry, and the antennal and behavioral responses of *Anopheles* mosquitoes, we identified and established the role of *Plasmodium* infection-associated compounds in human body odor.

Results

Attractiveness and *Plasmodium* infection

We measured the behavioral response of *Anopheles gambiae sensu stricto* (*s.s.*) to the foot odor of 5-12 year-old school children at two sampling time points, to assess whether *Plasmodium* infection changes the attractiveness of human hosts to mosquitoes. At time

point one (Fig. 1, T1), foot odor of asymptomatic *Plasmodium falciparum*-infected, and uninfected, children was collected on socks for 20 hours. For infected individuals, this occurred immediately after administration of the first dose of treatment with the antimalarial artemether-lumefantrine (AL), which is known to allow residual parasitemia during this time period (22). Odor samples were collected in the same manner from the same children 21 days later, following confirmed parasite clearance (Fig. 1, T2). Odor samples from participants with malaria parasites were categorized by those who harboured transmissible gametocyte stages (microscopy positive and/or more than 50 gametocytes/µL by the molecular diagnostic QT-NASBA, which detects female gametocyte *Pfs25* mRNA, *n*=23), or those with asexual stage parasites (microscopy negative for gametocytes and/or QT-NASBA gametocytes <50/µL, *n*=10). Samples were considered parasite-free (*n*=12) when no parasites were detected by microscopy and 18S qPCR (23). *Anopheles gambiae s.s.* mosquitoes were offered the choice of either T1 or T2 odor samples from the same child, in a dual choice cage assay (Fig. S1). The proportion of mosquitoes choosing the odors collected from children at T1 was significantly affected by parasitological status (GLM, F-test, $P<0.001$). Mosquitoes were more attracted to odors collected at T1 from children harbouring asexual or gametocyte stage parasites relative to T2 odor samples (GLM, 95 % confidence intervals (95 CI) 0.55-0.62 and 0.59-0.63 respectively, Fig. 1). Across both groups, the ratio of attraction to 'infected' (asexual or gametocyte carriers) versus 'parasite-free' odor was 0.6 to 0.4. Mosquitoes did not differentiate between T1 and T2 odor samples from parasite-free children, indicating that the difference observed between T1 and T2 odor was not an effect of sampling time point (GLM, 95 CI: 0.48-0.54, Fig. 1). This effect was independent of age, sex, tympanic (in-ear) temperature, or hemoglobin level at the first time point. These results indicate that infection with microscopically observable densities of either asexual stage parasites (median, 1340 [interquartile range, IQR: 480-2720] parasites/µL [p/µL]) or gametocytes (median, 80 [IQR: 40-680] p/µL) is associated with changes in odor profile that increase attraction to mosquitoes. This finding supports previous studies that demonstrate the heightened attractiveness of infected hosts, although here, by offering foot odor alone, we preclude the influence of other factors including breath. We did not observe the gametocyte-specific effect that was previously described (7–9), although we cannot rule out the possibility that low densities of gametocytes in some 'asexual' participants contributed to their increased attractiveness. To determine which chemicals in body odor are responsible for the observed differences in attractiveness, we repeat-sampled 56 *Plasmodium*-infected and parasite-free children from the same locality, using air entrainment to collect foot odor samples onto polymeric filters for further analysis.

Antennal response to malaria odor

We analysed air entrainment odor extracts using coupled gas chromatography-electroantennography (GC-EAG) (15). A change in the electric potential across the antenna resulting from stimulated neuropsychological activity, i.e. the EAG response, is caused

during olfactory nerve cell response. This allows detection of compounds to which the mosquitoes are potentially behaviorally active (Fig. 2). Point-of-care malaria diagnostics (rapid diagnostic test (RDT) and microscopy), used to inform odor sampling from asymptomatic individuals, were retrospectively confirmed using molecular diagnostics. Infected children were treated after odor sampling, and repeat sampling of all individuals was attempted one and three weeks later alongside repeat parasitological diagnoses (Fig. 2). Odor samples from individuals harbouring similar *Plasmodium* parasite stages or densities were extracted into solvent and mixed to create blends of 'average' odor with the following infection profiles: (1) *Plasmodium* infection, no gametocytes (2) *Plasmodium* infection, high-density gametocytes, and (3) parasite-free individuals (Table S2). A further group, (4) *Plasmodium* infection, sub-microscopic gametocytes, was included due to the frequency of sub-microscopic gametocytemia in endemic infections. *Plasmodium falciparum* gametocyte densities were determined by *Pfs25* mRNA QT-NASBA, while 18S qPCR and duplex qPCR were used to determine *P. falciparum* and *Plasmodium* densities respectively. Twenty-two analytes (Table S3) were found to elicit antennal response in *Anopheles coluzzii* (formerly the M-form of *An. gambiae s.s.* Giles), including the aldehydes heptanal, octanal and nonanal. No EAG-active analytes were specific to any of the infection profiles (1-4), indicating that any *Plasmodium*-induced change in the compounds used by host-seeking *Anopheles* must occur by variation in the relative amounts of compounds that are present in parasite-free individuals.

Plasmodium **infection-associated compounds (IAC)**

To investigate whether *Plasmodium* infection indeed results in quantitative changes in the production of volatile compounds, we compared the profiles of 117 foot, and 59 control (empty entrainment bag), odor samples. A total of 56 individuals participated in air entrainment odor sampling (Fig. 2), however, not all individuals were available at follow-up time points. Foot odor samples from *Plasmodium*-infected individuals were categorized by infection status: those from individuals with 'higher' (>50 p/µL, which approximates the microscopy limit of detection), and 'lower' (<50 p/µL), density infections, and those from individuals harbouring microscopic gametocytes ('total density' categorization). As the prevalence of non-*P. falciparum* infections was low (5.05 % [*n*=5] and 3.96 % [*n*=4] for *P. malariae* and *P. ovale* spp. respectively at day 0, and eight of nine had concurrent *P. falciparum* parasites), we did not separate samples from individuals with non-*falciparum* infections. Our analysis revealed increases in the production of the aldehydes heptanal, octanal, nonanal, (*E*)-2-octenal, and (*E*)-2-decenal by infected individuals. Increases were broadly associated with infections of high parasite density, relative to either low density, or production by parasite-free individuals. High density infections were also correlated with the presence of gametocytes in this dataset (Fig. S4C). Heptanal was produced in significantly greater amounts by individuals with higher parasite densities (>50 p/µL) relative to parasite-free individuals (REML, LSD, 5 %, Fig. 3A/C). Octanal and nonanal were produced

in significantly greater amounts by individuals with higher, relative to those with lower (<50 p/μL), density infections (REML, LSD, 5 %, Fig. 3D/3F/3G/3I). To investigate further this seemingly density-dependent effect, we divided the 'higher' and 'lower' density individuals into quartiles, representing 'low', 'medium-low', 'medium-high', and 'high' density. We observed a clear correlation between increased production of heptanal, octanal and nonanal, and increased parasite density (Fig.s 3B/E/H). The difference in production of nonanal between 'low', or negative, and 'high' individuals was significant (REML, LSD, 5 %, Fig. 3H, Table S6). Relative to parasite-free individuals, there was a trend for all *Plasmodium* parasite-positive individuals to produce more of the unsaturated aldehydes (*E*)-2-octenal and (*E*)-2-decenal (Fig. 3J/K), and for the latter, this difference was significant if individuals were categorized simply as *Plasmodium*-positive or parasite-free (REML, 'positive vs. negative' categories, LSD 5 %, Table S6). It is well-established that aldehydes are among the many volatiles that constitute human skin odor (24–26), where they are frequently cited as being predominant (27). Additionally, the ketone 2-octanone was found to be associated with the presence of microscopic gametocytes (REML, LSD, 5 %, Fig. 3L). Again, ketones are known volatiles of human skin. For all IAC, we found a quantitative relationship: the majority of individuals produced these compounds, but the quantity produced increased with *Plasmodium* infection. An average of 177 (standard error 5.23) analytes were captured per sample, and the IACs were disproportionately abundantly produced (Fig. 4), comprising on average 22.92 % of the total odor profile across all 117 samples. When production was ranked relative to all other compounds sampled, nonanal had a median rank of one, octanal two and heptanal five. While specific IAC were produced in greater amounts by individuals harboring parasites, an overall increase in volatile emissions from infected persons was not observed (REML, LSD, 5 %, Fig. S10), contrary to findings in the mouse or CHMI system (6, 21). Among the IAC, the antennal response, observed by GC-EAG to heptanal, octanal and nonanal, suggests that changes in the production of these compounds could affect mosquito behavior.

Mosquito response to IAC

To determine whether the IAC were attractive to mosquitoes, and therefore likely to be responsible for the increased attractiveness observed in infected individuals, we tested all six IACs (heptanal, octanal, nonanal, (*E*)-2-decenal, (*E*)-2-octenal and 2-octanone) in behavioral bioassays with *An. coluzzii*. We tested for a behavioral response towards the latter three compounds because of their positive association with the presence of parasites in the bloodstream (Fig. 3), despite a lack of antennal response in *An. coluzzii* (Table S3). First, the odor of parasite-free children (worn socks) was supplemented with the IAC individually, and tested at a minimum of two concentrations each. Of these, adding 10 μL of heptanal at 10^{-8} g/mL to parasite-free odor significantly increased attractiveness, relative to parasite-free odor alone (GLM, 95 CI: 0.60-0.84, Fig. 5), while heptanal at 10^{-7} g/mL had no effect. The attractive concentration is approximately $1/10^{th}$ of the additional heptanal

isolated in odor samples from individuals with 'higher' density *Plasmodium* infections, relative to negative individuals, over the corresponding time period. This suggests that elevated emission of heptanal, at specific concentrations, by parasitemic children could contribute to their increased attractiveness to mosquitoes. Supplementing with octanal, nonanal, (*E*)-2-decenal, (*E*)-2-octenal or 2-octanone alone did not induce altered behavioral responses, despite the EAG-activity observed in response to octanal and nonanal (Fig. S5). We then tested whether the addition of heptanal to a current best-practice synthetic mosquito lure, MB5 (comprising ammonia, L-(+)-lactic acid, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine (28)), might further increase attractiveness to mosquitoes. However, MB5 supplemented with heptanal was equally attractive as control MB5, at three concentrations (Fig. S5). This suggests that the attractiveness of heptanal observed with parasite-free odor was dependent on synergism with other volatile compounds naturally present, but absent from the synthetic MB5 blend. Because odor detection and response are highly contextual, this is not an unexpected outcome. To investigate further the behavioral role of IACs, but allowing for such synergistic effects between these compounds, we tested two blends with MB5: Plas 5 contained the aldehydes found to be associated with increased total parasite density (heptanal, octanal, nonanal, (*E*)-2-octenal and (*E*)-2-decenal), and Plas 6 additionally contained the ketone 2-octanone that was associated specifically with gametocytes. Each was tested at four concentrations. The Plas 5 blend enhanced attractiveness of MB5 (1 % concentration, GLM, 95 CI: 0.51-0.77, Fig. 5). However, the Plas 6 blend was not found to increase attractiveness of MB5 at any concentration (Fig. S5), which suggests that the gametocyte-associated 2-octanone moderated the attractiveness of the Plas 5 aldehydes. Given the presence of small amounts of 2-octanone in parasite-free odor, however (Fig. 3I), which increases in attractiveness on addition of heptanal (Fig. 5), it appears that this repellency of 2-octanone is not observed in the context of natural human odor. In previous studies describing the increased attraction of gametocyte carriers, the odor tested included both body and breath (7, 9), leaving open the possibility that the gametocyte-specific attraction may have originated in the breath. Indeed, 'malaria-associated' volatile compounds have been identified in the breath, whose production varied cyclically according to *P. falciparum* parasitemia, although no investigation of the mosquito response to those compounds was undertaken (29). Our behavioral tests show that supplementing parasite-free odor with heptanal increases attractiveness to mosquitoes. However, heptanal alone did not increase the attractiveness of a basic synthetic lure, while a blend of infection-associated aldehydes including heptanal (Plas 5) was attractive. Therefore, in both instances, the increased attraction was dependent on additive effects among the infection-associated aldehydes, which are naturally present in 'parasite-free' odor at lower concentrations (Fig. 3).

Discussion

Aldehydes are found in the skin odor of various mammalian species (30), and have previously been determined to be among the chemicals used by hematophagous insects for host location (31). These oxygenated compounds can be synthesized when reactive oxygen species attack lipid-dense membrane structures (32), i.e. lipid peroxidation, caused by oxidative stress. Oxidative stress is known to characterize malaria infection (33), occurring in the erythrocytes and liver. Alternatively, or additionally, the aldehydes found here may have been produced directly by *Plasmodium* parasites: a recent publication found the aldehydes octanal, nonanal and decanal to be among volatile compounds emitted by red blood cell (RBC) cultures that had been supplemented by (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (34). HMBPP is a precursor in the 2-*C*-methyl-*D*-erythritol 4-phosphate (MEP) pathway, apparently used by *Plasmodium* for isoprenoid production, and it was suggested that HMBPP triggered enhanced release of these compounds from infected RBC, with a subsequent impact on mosquito attraction. Additionally, terpenes were isolated from HMBPP RBC, and another study also isolated terpenes above *Plasmodium* infected RBC cultures (35). Although the MEP pathway is a possible source of terpenes via isoprenoid production in infected RBC (35), the source of terpenes in HMBPP RBC remains unknown (34). We did not find an association between *Plasmodium* infection and the emission of terpenes from the skin, corroborating earlier findings in *Plasmodium*-infected mice (6). It should be emphasized that laboratory-based studies of the volatile compounds isolated above iRBC cultures do not characterize the human body odor used by mosquitoes during host location. As such, they do not fully capture the complex biological and biochemical host-parasite interactions that occur in natural *Plasmodium* infections. In our study, the production of aldehydes was increased in individuals with *Plasmodium* infection. The extent to which parasite-specific release of aldehydes from iRBC would contribute to a profound and systemic increase in aldehyde production, caused by malaria-induced oxidative stress, remains unexplored. Finally, it is important to note that while the lipid peroxidation pathway for aldehyde production is well-established, the skin microbiota is also known to produce aldehydes. This is particularly relevant to our study, as odor samples were taken from the feet. Feet harbor skin microflora that produce volatiles that are attractive to mosquitoes (36), and differences in microflora have been associated with differences in attractiveness (16).

We demonstrated that elevated production of specific aldehydes in skin odor is associated with increased attractiveness to mosquitoes in *Plasmodium*-infected people. Our findings are in accordance with the 'deceptive signaling' hypothesis, whereby host cues already used by host-seeking insects are exaggerated, increasing the attractiveness of that vertebrate to biting insects, but when the blood-meal is in fact unfavourable to the insect (37). If the disadvantages (e.g. reductions in fecundity (38–40)), shortened lifespan (41–43)) of taking an infected blood meal outweigh any advantages (e.g. reduced host defenses (2), faster

engorgement (44)), the evolution of an infected-host avoidance phenotype might be expected. *Anopheles* may less easily select against an infected-host phenotype comprising 'normal' stimuli. It is possible that the observed changes to skin odor are specific to *P. falciparum*, which constituted the majority of infections in the cohorts that we studied. In similar studies of mosquito attraction, increases are often associated with the chronic phase of malaria, and/or increased density of bloodstream gametocytes (2, 5–9). We found that odor from all *P. falciparum*-infected individuals was more attractive than that of parasite-free individuals, and the increased production of IACs was correlated with total parasite density. Although it is possible that gametocytes at densities below the detection limit of our assay (45) contributed to the attractiveness of individuals with asexual malaria parasites, the greatest production of IAC did not correlate with gametocyte density. The association between *P. falciparum* asexual parasite biomass and gametocyte density is generally positive (46–48), and we also observed this in our study. Thus, our findings are in broad agreement with studies that observed an increase in attractiveness related to the presence of gametocytes (2, 5–9) or general malaria infection (4).

The compounds we observed to be associated with infection could be derived from malaria-induced oxidative stress. Although there is *in vitro* evidence that suggests both octanal and nonanal could be produced by red blood cells via interactions with components of the isoprenoid production pathway (34), found in *Plasmodium* parasites, both this and the oxidative stress mechanism would result in the observed correlation between increased parasite density and increased compound production. More generally, this correlation would be observed if IAC were the by-product of any sequelae of *Plasmodium* infection, with the resulting influence on mosquito behavior a coincidental benefit to the parasite. This raises the question of whether the IAC found in our study are specific to *Plasmodium* infection, or could be a general 'scent of infection'. Indeed, increased aldehydes have been found to signify the presence of other diseases (32, 49, 50). However, our data do indicate that the observed increase in these IAC is specific to *Plasmodium* infection, because a general 'scent of infection' might have been expected in malaria parasite-free children who harbored other infectious organisms, and as such there would have been no observed difference in IAC production between the uninfected and infected children in this study. For example, *Schistosoma mansoni* was recently found in 51% of 9 to 12 year-old children in neighboring Asembo District (51), and was likely present in individuals in our malaria parasite-free cohort. To date, only one study on the effects of *Plasmodium* infection on volatile emission in humans included a control group of bacteria-infected participants, and they found that increased levels of thioethers in human breath were indeed malaria specific (29). Nevertheless, an important follow-up to our study would be to examine skin odour profiles, and behavioral responses of mosquitoes towards these, of individuals affected by other diseases thought to be characterised by similar emissions but not vectored by an insect.

Although it is not possible to infer from this study whether the increased production of IAC is under the control of malaria parasites, it could be argued that if the parasites indirectly stimulated compound production (e.g. via triggering oxidative stress), the parasite genes underlying this stimulation would be selected for via enhanced transmission. However, a mutation benefitting transmission may be costly to the parasite and would therefore only be selected if the trade-off resulted in a net increase in transmission. Further studies that explore the relative costs and benefits of manipulation to the parasite, for example through modelling (52), are merited. The implications of identifying infection-associated compounds, with their demonstrated impact on mosquito behavior, are far-reaching: we better understand parasite-vector-host transmission events, and their over-dispersed nature in human populations. These compounds may permit further improvement of already highly functional lures for trapping malaria mosquitoes, or even serve as biomarkers for malaria, providing a basis for novel and non-invasive diagnostic tools.

Materials and methods

Ethics

Study participants were five- to twelve-year-old children local to the Thomas Odhiambo Campus of *icipe* in Western Kenya (0°25'48.1"S, 34°12'24.5"E), including Rusinga Island, in Suba District, Homa Bay County. Participants were recruited after obtaining signed consent. The study protocol (NON SSC 389) was approved by the Scientific and Ethical Review Committee of the Kenya Medical Research Institute (KEMRI/RES/7/3/1). Subsequent analyses were conducted at the London School of Hygiene & Tropical Medicine (LSHTM) (ethics reference 8510).

Attractiveness of 'infected odor' (socks) by cage assays

A cohort of *Plasmodium*-infected, asymptomatic (tympanic temperature <37.5 °C), individuals that participated in an olfactometer study (9) was studied for the attractiveness of their skin odor to *Anopheles gambiae s.s.* Forty-five children were included, of which there were: 23 with microscopic gametocytes or an estimated gametocyte density above 50 gametocytes/µL blood by QT-NASBA, 10 positive for asexual parasites but not gametocytes by microscopy, and 12 that tested *Plasmodium*-parasite free by 18S-qPCR (23). Samples were collected at two time points: within 24 hours of antimalarial treatment but while children still harboured parasites (22) (time point one [T1] samples), and 21 days later (time point two [T2] samples). Antimalarial treatment with artemether-lumefantrine (AL) was administered to *Plasmodium* positive individuals according to manufacturer's instructions (20 mg artemether/120 mg lumefantrine per tablet, Coartem™; Novartis, Basel), and socks were put on within one hour of treatment. Age, hemoglobin (Hb), weight and temperature were measured as covariates (9). At day 21 ('after'), both parasitological testing and participant covariate measurements were repeated.

Procedures for collection of body odor

Body odors were collected for 20 hours on nylon socks (97 % polyamide, 3 % elastane, 20 denier, Hema, The Netherlands), which were washed using 70 % ethanol and dried at 70 °C for two hours before use. Surgical gloves were worn throughout collection procedures. Children were assisted in putting on and removing the socks. These were stored in clean glass jars at -20°C until use in cage assay experiments. Children were asked not to bathe during this time but had no other behavioral restrictions.

Behavioral assays for attractiveness of odor samples

A dual choice cage assay was modified (53) to determine the relative attractiveness of odor samples from T1 and T2. Three WHO bioassay tubes (12.5 cm long, 5 cm wide) (54) were connected with sliding units between the inner and outer tubes (Fig. S1). Mosquito cages (15×15×15 cm) were wrapped with transparent kitchen cling-film (Chandaria industries Ltd., Kenya), to prevent movement of volatiles between different assays running in parallel. The outer tubes were inserted six cm into the cages. Per individual, T1 and T2 samples (sock pairs) were placed in opposing cages, with the feet cut off to remove environmental soiling.

Six- to eight- day old, non blood-fed, female *An. gambiae s.s.* mosquitoes (Mbita strain, with published rearing methods (55)) were collected prior to the experiment and allowed eight hours acclimatization. Ten mosquitoes were released into the central tube per bioassay, and the gates of the tubes opened for 15 minutes to allow mosquitoes to make a choice of odor source. Experiments were conducted between 18:30-22:30 under ambient conditions, in a red fluorescent-lit room (average temperature, 24.1 °C) with the dual cage covered by black cotton cloth. After 15 minutes mosquito choice was recorded. All sock pairs were tested simultaneously on the same nights, and in total each pair (child) was tested six times, replicating over experimental nights, dual cage set-ups and between cages. All disposable equipment was changed, and cages cleaned (70 % ethanol), between experiments/replicates.

Statistical analysis

Per child, the number of mosquitoes that chose the T1 or T2 odor sample was summed over six replicates, and the relative attractiveness of samples determined as the proportion of mosquitoes that selected a sample over the total number of mosquitoes that made a choice. A generalized linear model (GLM; Binomial distribution, logit link function and dispersion estimated) was used to test the effect of parasitological status (parasite free, asexual or gametocytes) on the relative attractiveness. The number of mosquitoes caught in the cage with the T1 sample was used as the response variable, and all mosquitoes caught in both cages as the binomial total. Covariates associated with participants (age, sex, Hb and tympanic temperature measured at T1) were tested, but removed from the model because they were not significant (P>0.05, F-tests). Per parasitological group, we used the 95 CI of the predicted proportion of mosquitoes choosing T1 odor samples, derived from the GLM, to assess whether mosquito choice differed significantly from a 50:50 distribution over the two odor samples. SPSS® (2016, version 24, IBM) was used for the analyses.

Collection of volatile odor samples

In the same locality, a separate cohort of schoolchildren, of varying *Plasmodium* infection status, were sampled for foot odor (Fig. 2, top half). On day zero, twenty children for whom the parent or guardian had given full consent were tested for their malaria status by rapid diagnostic test (RDT) and microscopy. Tympanic temperature, age, weight and Hb levels were recorded. Symptomatic children and/or those with a temperature >37.5 °C with RDT positivity were treated with AL (as above), and excluded from the study. Overnight, microscopy was conducted and three children were selected for odor sampling, with the intention to sample one child with asexual parasites, one with gametocyte stages, and one with no parasites. On day one, odor sampling was conducted by air entrainment, after which all malarious children were treated. Days zero and one constituted round one (R1), and the same procedures were conducted at days seven and eight (R2), and 21 and 22 (R3), with the intention to repeat sample the same children at two points post-treatment (Fig. 2). R1-R3 were repeated for six months between January and June 2014. In this way, 56 children were repeat sampled, but a total of 117 odor samples, and 59 accompanying empty bag control samples, was achieved, due to loss-to-follow-up.

For each child, one foot was placed in a prepared bag (Fresh and Eazy oven bags, 45 x 50 cm, Meda-Pak, Uithoorn, The Netherlands), clipped shut around the calf. At each sampling round (R1-R3), a control (empty) bag was tightly closed and sampled in the same manner. Bags were fitted with Swagelok fittings at opposing corners, allowing connection to polytetrafluoroethylene (PTFE) tubing for air flow. Air (charcoal-filtered) was pumped into the top of the bag and vacuumed from the bottom (both at 500 mL/min), with a 30-minute purge prior to fitting the polymer filters, to ensure system cleanliness. Porapak filters were connected (Porapak Q, mesh size 50/80, Supelco Analytical, Bellefonte, PA, USA) and sampled for 100 minutes, then stored in stoppered glass vials in a cool box before sealing under filtered nitrogen on the same day. Ampoules were stored at -20°C until shipping to LSHTM. Prior to use, all PTFE tubing, Swagelok fittings and glassware were cleaned with 70 % ethanol, then baked in an oven at 150 °C for two hours. Sampling bags and charcoal filters were baked in the same manner. Cotton gloves were worn by the investigators throughout.

Infection status

Odor sampling was informed by RDT (One Step malaria HRPII and pLDH antigen rapid test [SD BIOLINE, Cat no 05FK60]), performed as per manufacturer's guidelines, and thick and thin blood films made using peripheral blood from a finger prick. Whole blood (50 μL) was stored in RNAprotect (250 μL; QIAGEN, Germany). Retrospectively, DNA/RNA extraction was performed using Total Nucleic Acid Isolation Kit (with methods as published previously (56)) and *P. falciparum* parasite density, and stage V gametocyte density, determined by 18S qPCR (23) and QT-NASBA (57). Additionally, dried blood stored on both Whatman No. 3

filter paper (Whatman, Maidstone, United Kingdom [wDBS]) and used RDTs (air dried and stored in sealed plastic bags containing the desiccant silica gel [uRDT]) was used as a DNA template. DNA was extracted from circles (3 mm) punched from the wDBS, and sections (3 x 2 mm) cut from the central section of the nitrocellulose strips in the uRDTs (58). Extraction was performed in a deep well plate using an automated extraction system (QIAsymphony), with the QIAsymphony DSP DNA mini kit (QIAGEN, Germany) and according to the manufacturer's instructions, and a *Plasmodium* tRNA methionine-based duplex qPCR was used to measure *Plasmodium* density (22). Good correlation in parasite density was obtained between duplex qPCR using wDBS or uRDT whole blood template (59). Where available, the same DNA extracts were used for species specific (*P. falciparum, P. ovale* spp. and *P. malariae*) nPCR (60), with some *P. ovale* spp. identifications confirmed by the *P. ovale* spp. tryptophan-rich antigen (PoTRA) assay (61).

Gas chromatography-electroantennography (GC-EAG) of pooled odor samples

GC-EAG odor sample blends

Porapak filters were eluted using re-distilled diethyl ether (750 µL), and, to approximate an "average" odor per category, extracts were pooled according to the individual's parasitological status: (1) *Plasmodium* infection, no gametocytes (2) high-density *P. falciparum* gametocytes (3) parasite-free individuals, (4) *Plasmodium* infection, sub-microscopic *P. falciparum* gametocytes (Table S2). Aliquots (400 µL) of extracts were mixed, then concentrated (to 60 µL) under a stream of nitrogen (charcoal-filtered). Glassware, charcoal filters and PTFE tubing were cleaned as before.

Experimental set-up

GC-EAG was conducted during the scotophase, using four- to eight- day-old, unfed female *Anopheles coluzzii* (N'gousso strain (62)). Adults were maintained at 70 % RH, with a 12 h light/dark cycle (scotophase 09:00 – 21:00) and access to 50 % glucose solution. The order of testing blends was determined by a 5 x 5 Latin square (including control blend). The mosquito head was dissected, and the palps, proboscis, and half of the terminal (13^{th}) antennal flagellomere cut off. The indifferent electrode was inserted into the back of the head and the antennal tips guided into the recording electrode to complete the circuit (Fig. 2). Electrodes were hand-pulled glass tips inserted over silver wire (diameter 0.37 mm; Harvard Apparatus, Edenbridge, UK) and filled with Ringers' solution (15). Gas chromatography (GC) was performed on a 7890A machine (Agilent Technologies®), with the following program: oven temperature maintained at 40 °C for 0.5 minute, increased by 10 °C per minute to 230 °C, then held for 20 minutes. Blends were injected at 4 µl, and the eluate was split to the FID detector and EAG interface at a ratio of 1:1. At the EAG interface, the eluate passed from the heated splitter column to a stream of charcoal filtered,

humidified air (flow rate 400 mL/min). This airflow was directed over the antenna at a distance of 5 mm. The signal was amplified x10,000 by the Intelligent Data Acquisition Controller-4, and signals were analyzed using EAD 2000 software (both Syntech®, Hilversum, The Netherlands). Responses were signified by a depolarization of sufficient amplitude. Peaks that elicited responses in more than 3, of the 6/7 total repetitions, were considered to be EAG-active.

Analysis of odor profiles by GC

Instruments used for GC analysis were 7890A, 6890N and HP6890 (Agilent Technologies, Stockport, UK). Each was fitted with a cool-on-column injector, flame ionization detector, used hydrogen carrier gas, and 1 µL injections were performed. All were fitted with an HP1 column, 50 m x 0.32 mm, film thickness 0.52 µm, and the following program was used: oven temperature maintained at 40 °C for 0.5 minutes, increased by 5 °C per minute to 150 °C, held for 0.1 minute, raised by 10 °C per minute to 230 °C, held for 40 minutes. Traces were analyzed using the R package MALDIquant (63) (R version 3.3.0, 2016, The R Foundation for Statistical Computing©). In brief, raw *x*,*y* co-ordinates for GC traces were exported from Agilent ChemStation (C.01.04) and the *y* value (height, for 1 µL) multiplied by total extract to represent actual amount per sample (ng). Following baseline removal, traces were visually inspected for consistent differences between parasitological groupings. Compounds of interest (COI) were then compared quantitatively, by integrating peaks in ChemStation, and calculating retention index and amount relative to a standard series of n-alkanes (C7-C25), using Equation 1.

Equation 1. Retention index (RI) calculation

$$RI = 100\,((\log_{10}RtX - \log_{10}Rtn))/(\log_{10}Rtn+1 - \log_{10}Rtn)) + 100n$$

RtX = Retention time for compound of interest
Rtn = Retention time for alkane before compound of interest
Rtn+1 = Retention time for alkane after compound of interest
n = number of carbons in alkane before compound of interest

Following statistical analysis (below), IAC were tentatively identified by gas chromatography-mass spectrometry (GC-MS), using either a Micromass Autospec Ultima (a magnetic sector mass spectrometer equipped with a Programmed Temperature Vaporizing inlet (GL Sciences B.V., Eindhoven, The Netherlands) and Agilent 6890N GC), or a Mass Selective Detector (quad GC-MS). Peaks were compared with MS databases (National Institute of Standards and Technology, NIST). For confirmation of identification, authentic standards were injected onto two GC columns (HP1 and DB wax) simultaneously with samples containing those compounds. Standards were: heptanal (Sigma-Aldrich), octanal (Sigma-Aldrich), nonanal (Sigma-Aldrich), (*E*)-2-octenal (Acros Organics), (*E*)-2-decenal

(SAFC), 2-octanone (Sigma-Aldrich). Identifications were considered certain when the resultant peak increased in height without increasing in width. Co-injections were conducted for all IAC.

Statistical analysis

Any sample that had detectable parasite DNA at amounts greater than published limits of detection (LOD) for the assays (0.02 p/µL for 18S (23) and 5 p/µL for duplex qPCR (22)) was considered positive, and those with DNA amounts beneath these thresholds were excluded. Only samples that were negative by all measures, including at least one molecular diagnostic measure, were taken to be negative, other than RDTs for which positivity was acceptable (on an assumption of positivity due to circulating HRP-2 protein) (64). Individuals with *Plasmodium* parasitemia, but without microscopic gametocytes, were divided into higher and lower parasite density categories: 'higher density' with greater than 50 p/µL, and 'lower density' with between the LOD and 50 p/µL (Fig. S4A). Categorization was informed using 18s qPCR, then duplex qPCR (wDBS>uRDT), then microscopy, according to assay result availability. Instances suggesting no parasites by 18S qPCR but with a robust parasite signal from one or more other measures were allocated to the appropriate positive category. For 'quartile' categories, 'higher' and 'lower' density samples (*n*=81) were then subdivided into quartiles according to density (Fig. S4B). Again, samples were allocated according to a hierarchy of procedures, in the order 18S qPCR > duplex qPCR > microscopy. Where 18S and/or duplex qPCR result was zero or missing but microscopy was positive, the film was re-read and that value assumed. Two samples with low parasite density by 18S but high and corresponding density by duplex qPCR and microscopy were allocated according to the two corresponding outcomes, and one further 'lower density' sample was excluded from 'quartile' analysis due to imprecise parasite density. Gametocyte densities per group, 'total density' categories, are given in Fig. S4C (measured QT-NASBA, where available), and the correlation between 18S qPCR and duplex qPCR by two templates in Fig. S4D.

The association between the production of COI (variate: percentage of total entrainment) and parasitological category was assessed by linear mixed models fitted using the method of residual maximum likelihood, REML. This modelling allowed for unequal sample sizes (per parasitological category) and repeated measures on the same individuals. We tested (F-tests) for the main effects of covariates (age, Hb, day of the year, weight) before the treatment (parasitological status) term, and for factors (sex, round) after the treatment term, in a forward selection, parallel-lines, regression analysis approach (Table S6). Pairwise comparisons between groups of most biological interest were made using the LSD at the 5 % level (Table S7), and COI demonstrating significant (REML, LSD, 5%) differences between groups were termed 'infection-associated compounds'. Data analysis was conducted using Genstat (2013, 16th edition, VSN International, Hemel Hempstead, UK).

Behavioral testing of candidate compounds

Testing IAC individually

Six IAC (heptanal, octanal, nonanal, (*E*)-2-octenal, (*E*)-2-decenal and 2-octanone) were tested in a background of odor from the worn nylon socks of twelve parasite-free children (18S qPCR confirmed). Each sock pair was cut into twelve strips after removing the foot part, then 12 bundles were made, each containing a strip from each individual. Bundles were stored at -20 °C until, and between, experiments. IAC were positioned downwind and separated from sock bundles by a metal grid, ensuring no contact. Parasite-free odor (bundles) was tested with or without individual IAC (in 10 µL hexane on filter paper) and against the same but with hexane alone. For each IAC, a decimal dilution series was made (in hexane) and two/three concentrations chosen, to bracket the differential amount between significantly different groups (LSD 5 %, REML), adjusted to represent 15 minutes of compound release (test duration).

Improving a mosquito lure

Next, we verified whether the IAC could improve a mosquito lure for monitoring or mass trapping of *Anopheles*. Heptanal, the most promising candidate from the above experiment, was tested as well as two blends: Plas 5 contained the IAC that were associated with parasitological positivity (nonanal, heptanal, octanal, (*E*)-2-decenal and (*E*)-2-octenal), and Plas 6 additionally contained the gametocyte-associated 2-octanone (Fig. 3). Ratios were derived, and amounts of compounds were taken (Table S8) from predictions for compounds for parasitological groups with significantly increased quantity (LSD 5 %, REML). Plas 5 and Plas 6 were tested with the synthetic lure MB5 (28) at four concentrations, each decreasing by a factor of 10 from the 100 % concentration (Table S8).

Assay

A triple chamber dual-port olfactometer (65) was used to test the preference of 30 five- to eight-day-old female, non blood-fed *Anopheles coluzzii* (Suokoko strain, rearing procedures as published previously (21)) for parasite free odor or MB5, supplemented with IAC or IAC blends, against background odor alone (parasite-free odor or MB5). Mosquitoes were maintained in a release cage prior to testing (for 24 hours). Experiments took place during the last four hours of the scotophase under near-dark conditions (<1 lux). Mosquitoes were allowed to fly for 15 minutes, then those that had entered the traps with test/control odors were counted. Each IAC/concentration combination was tested eight-nine times on different days, and each Plas concentration 10-11 times on different days, rotating treatments between the left and right port of the olfactometer. Climatic data (R.H.,

temperature and air pressure) were recorded in the flight chambers and in the surrounding room.

Statistical analyses

Generalized linear models (GLMs) were used to test the effect of odors (individual IAC/heptanal/Plas blends) on relative attractiveness (the proportion of mosquitoes selecting the test odor). GLMs were run as described above (*statistical analysis*, attractiveness of 'infected odor' (socks) by cage assays), testing parameters associated with the set-up as additional factors or covariates in the model, and retaining when significant ($P<0.05$, F-test). Sets of compounds were run in separate models (Table S9). SPSS® was used for the analyses.

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Author contributions:

W.T., J.G.L. and R.C.S. conceived the study. A.O.B. conducted and oversaw odor sample collection, and conducted and analyzed the sock cage assays. J.G.dB. oversaw and assisted in conducting, and execution, of all aspects of the study, and specifically designed,

conducted and analyzed the IAC behavioral assays alongside M.A.V. The duplex PCR was overseen by K.B. and C.S., J.M. conducted the wDBS and uRDT DNA extractions, J.C. conduced the G.C-M.S. with A.R., and together with J.A.P. provided chemical input. P.W. advised and assisted in analysis of G.C. data with MALDIquant, D.M. and W.R.M. provided assistance at *icipe*, Kenya, R.S. advised on parasitology, and T.B. assisted and advised on parasitology and data interpretation. N.O.V. advised throughout the project and with specific attention to the entomology, and S.J.P. analyzed G.C. data and advised on statistical analysis. A.R. executed and analyzed the odor study (skin chemistry), the duplex PCR, nested PCR, and designed the figures. A.R., J.G.dB. and J.G.L. wrote the paper, and all authors reviewed the manuscript.

Additional information

The authors declare they have no competing financial interests.

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Figures

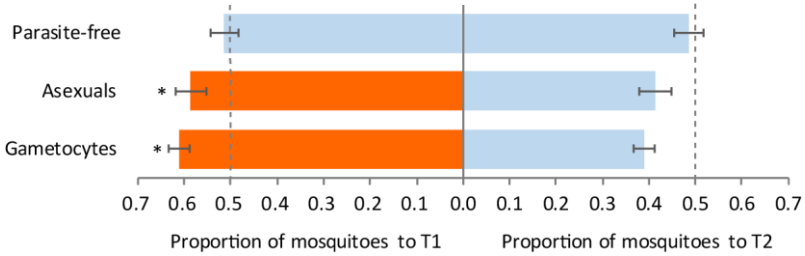


Fig. 1.

Effect of parasitological status on *Anopheles gambiae sensu strictu* (*s.s.*) preference for body odor sampled at two time points, one during *Plasmodium* infection (T1) and the other following parasite clearance (T2). Blue bars represent attraction to odor from parasite-free samples, orange bars represent attraction to odor samples from individuals with parasites. Groups of ten mosquitoes were given a choice between socks worn by each participant at both T1 and T2, in a dual choice cage assay, with the number of mosquitoes that chose the T1 or T2 odor sample being summed over six replicates per participant. Participants were grouped into those with gametocytes by microscopy or QT-NASBA (at >50 gametocytes/µL) (*n*=23), those with asexual stages only by microscopy (*n*=10), or parasite-free (*n*=12). Of those with asexual parasites, three had sub-microscopic gametocytes (1-34.9 gametocytes/µL blood), and three were not tested. Predicted mean proportions from the GLM are plotted with 95 % CI, and significant differences from 0.5 are indicated with * (*P*<0.05) (GLM included infection status only as predictor of proportion of mosquitoes attracted to T1 odor samples).

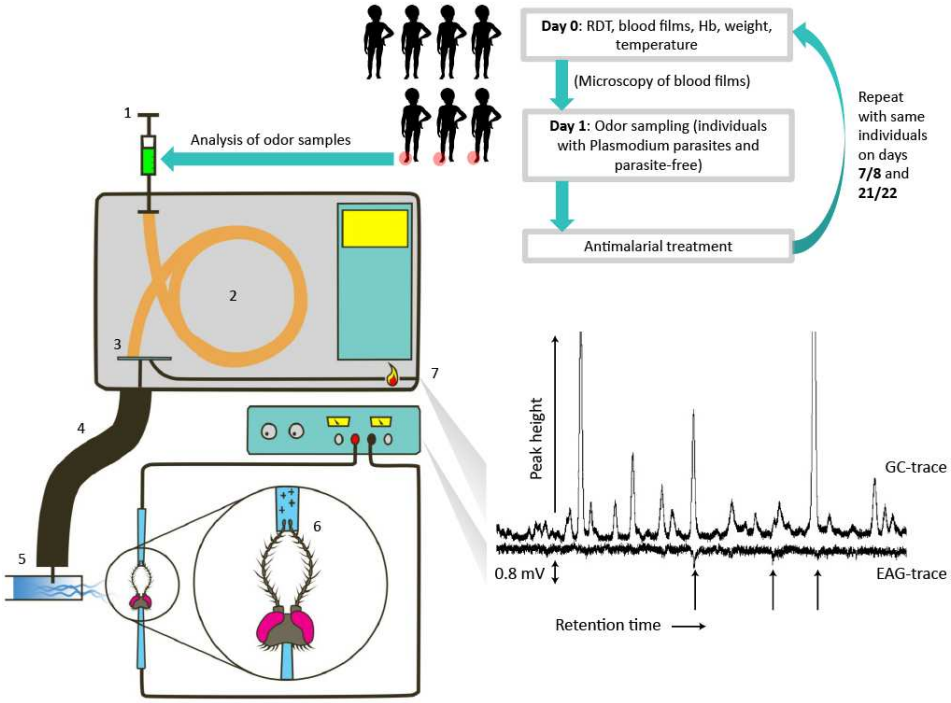


Fig. 2.

Schematic of protocol (top half of image) for odor sampling by air entrainment from *Plasmodium*-infected individuals, for use in GC-EAG analysis (bottom half of image, here with *Anopheles coluzzii*) and direct GC analysis of entire odor profile. Children were recruited for odor sampling in groups of three to represent parasite-free, asexual parasite carriers, and gametocyte carriers, if parasite prevalence allowed. Following malaria diagnosis by point-of-care methods and odor sampling, malarious individuals were treated, and the same cohort re-sampled on days 8 and 22. Whole blood samples were also taken for retrospective molecular analysis. During GC-EAG, odor samples are injected by syringe at the inlet directly into the column (**1**), where they are vaporized, and carried through the column by the carrier gas (here hydrogen) (**2**). During passage through the (50 m) HP1 column, constituents of the sample are separated by gas chromatography, and analytes are split as they elute from the column (**3**). A proportion is directed, via a heated transfer line (**4**), into a humidified, purified, airflow (**5**), which is then directed over the insect antennae (**6**), simultaneously to the proportion that is detected by a flame ionization detector on the GC (**7**). GC analytes are represented by peaks (top; GC trace) while antennal response by nerve cell depolarization causes a perturbation in the electroantennographic detection (EAG trace), indicating entomologically significant analytes. Image courtesy of Iain Robinson.

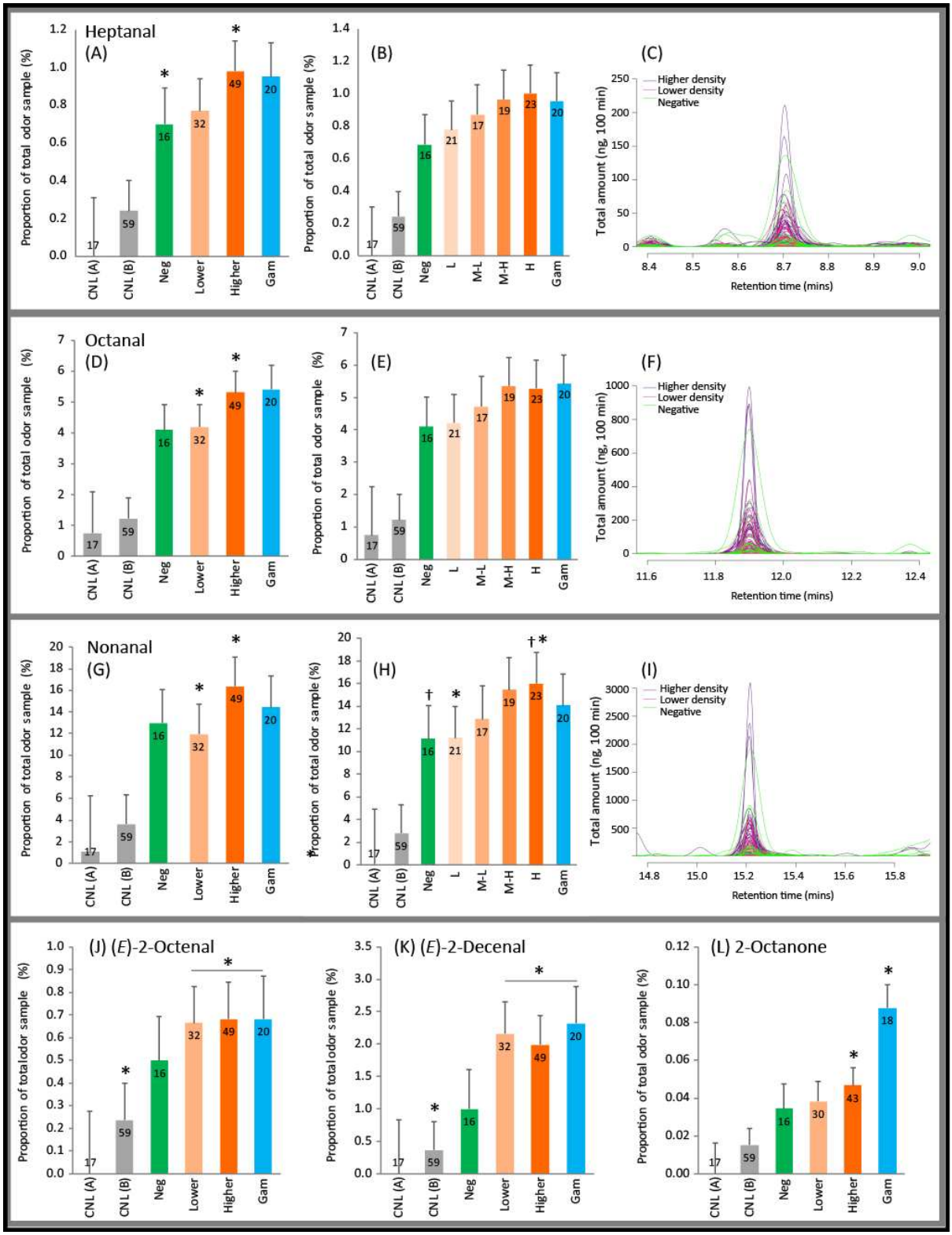


Fig. 3.

Amount of infection-associated compounds produced by individuals of differing parasitological status. (**A**)/(**B**)/(**C**) heptanal; (**D**)/(**E**)/(**F**) octanal; (**G**)/(**H**)/(**I**) nonanal; (**J**) (*E*)-2-octenal; (**K**) (*E*)-2-decenal; (**L**) 2-octanone production (relative to all compounds in odor sample) per group (100-minute odor profile sampling). Predicted means (+SE) given by

linear mixed modelling (REML). See Table S6 for details of the models and Table S7 for standard error of the difference (SED) values for comparison of predicted means. Sample size in bar ends, *****,**†** significant pairwise difference in mean amount between two groups indicated, tested by Least Significant Difference (P<0.05). **A, D, G, J, K,** and **L**, 'total density' categorization: 'Neg'=negative, 'lower' and 'higher' refer to parasite densities of lesser or greater than 50 p/µL, 'Gam'=microscopic gametocytes (**B, E, H**) 'quartile' categorization; 'Neg' and 'Gam' as before, L=low, mean/median parasite density 0.38/0.3, n=21; M-L=medium-low, mean/median parasite density 16.77/8.3, n=17; M-H=medium-high, mean/median parasite density 296.60/214.18, n=19; H=high, mean/median parasite density 102669.46/13304.54, n=23. For bar charts CNL(A)=solvent control, CNL(B)=empty bag control. **(C)**/**(F)**/**(I)** show raw gas chromatography output for heptanal, octanal and nonanal. Individual traces represent odor samples, colored according to the parasitological status of the individual from whom the odor sample was taken, 'Higher density', 'lower density', and 'negative' definitions as above. Gametocyte carriers are excluded for clarity, as compound production spanned higher and lower parasite density groups.

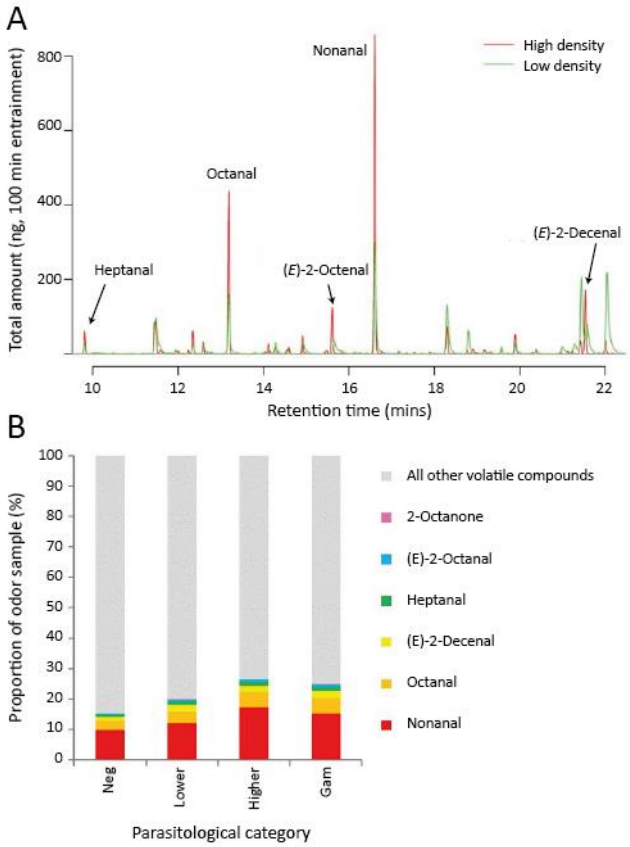


Fig. 4.

Comparison of odor profiles from parasite-free individuals, versus those harboring bloodstream parasites. (**A**) Representative GC traces from an individual with a 'high density' infection (>50 p/µL blood) and 'low density' infection (<50 p/µL blood). Compounds found to be associated with infection (other than 2-octanone, not visible due to very small amounts) are annotated. (**B**) The proportion (%) that IAC contributed towards the entire odor profile, grouped by parasitological category ('total density' categories). The average number of non-IAC per group (i.e. 'all other volatile compounds', grey bar), was 171.27 (SE=5.23) across all groups.

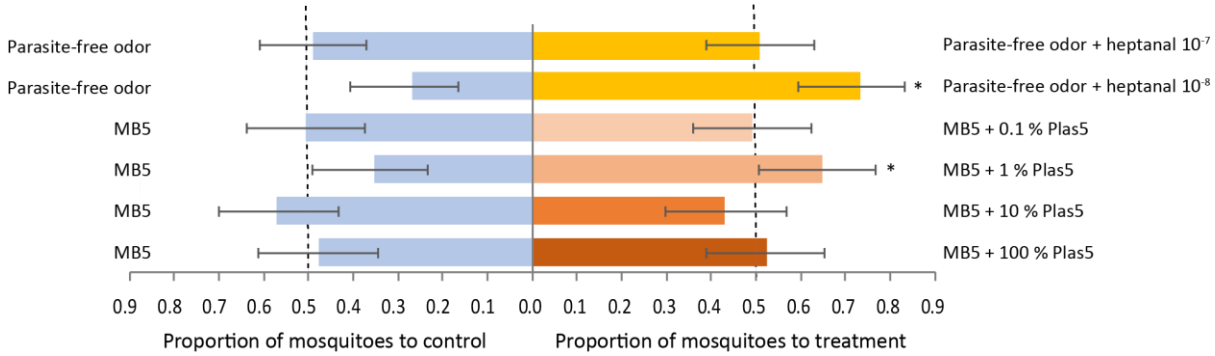
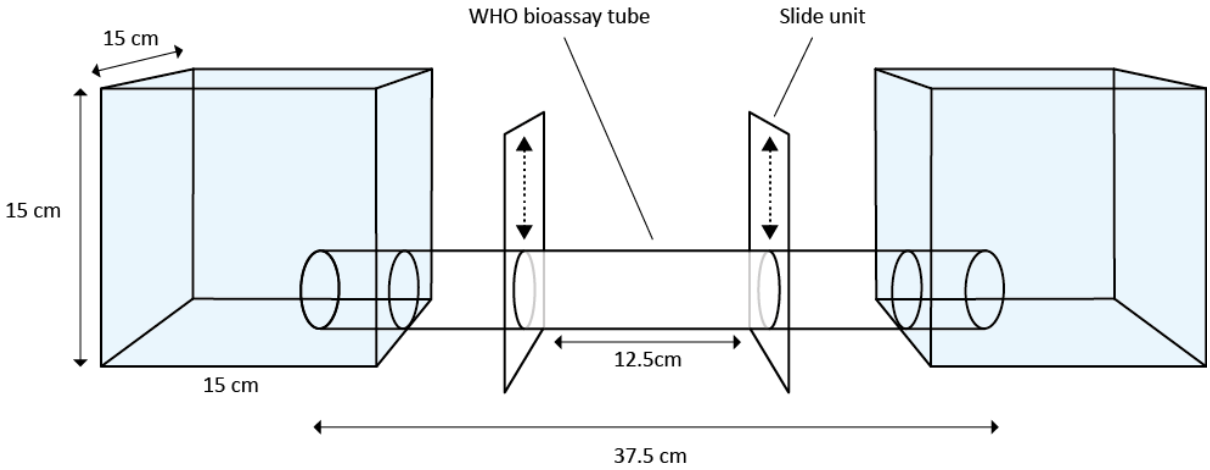


Fig. 5.

Anopheles coluzzii responses in a dual-port olfactometer to heptanal and a blend of five infection-associated aldehydes, 'Plas 5'**.** Heptanal (10 µL) at two concentrations (g/mL) was presented with (yellow bars), and tested against (blue bars), odor (socks) from parasite-free study participants (5-12 year-old Kenyan children) over eight replicates. Plas 5 (heptanal, octanal, nonanal, (*E*)-2-octenal and (*E*)-2-decenal) at four concentrations (10 µL of 100 % approximating the amounts found in the foot odor samples) was presented with (orange bars), and tested against (blue bars), the synthetic lure MB5 (ammonia, (S)-lactic acid, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine) over 10/11 replicates. Each replicate tested 30 mosquitoes. Predicted mean proportions and 95 % CI are presented, from two separate GLMs (for heptanal and Plas 5 assays), assuming a Binomial distribution and using a logit link function. Significant differences from 0.5 are indicated with * (P<0.05) (See Table S9 for details of the GLMs).

Supplementary information



S1. Schematic drawing of the cage assay used to test the effect of parasitological status on *Anopheles gambiae sensu stricto* (*s.s.*) preference for body odor sampled at two time points, one during *Plasmodium* infection (T1) and the other following parasite clearance (T2). Two mosquito cages wrapped with kitchen cling-film were connected using three WHO bioassay tubes[1], with slide units between the inner and outer tubes. Each cage contained a pair of socks, with samples from the same child, collected during infection or after antimalarial treatment, offered in a dual-choice situation. Ten female mosquitoes were released in the central tube and given 15 minutes to fly to either cage.

1. WHO. *Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets*. (2006). doi:Ref: WHO/CDS/NTD/WHOPES/GCDPP

S2. Parasitological information on GC-EAG blends, Kenya cohort. A control group was also tested, comprising 10 control (empty bag) samples.

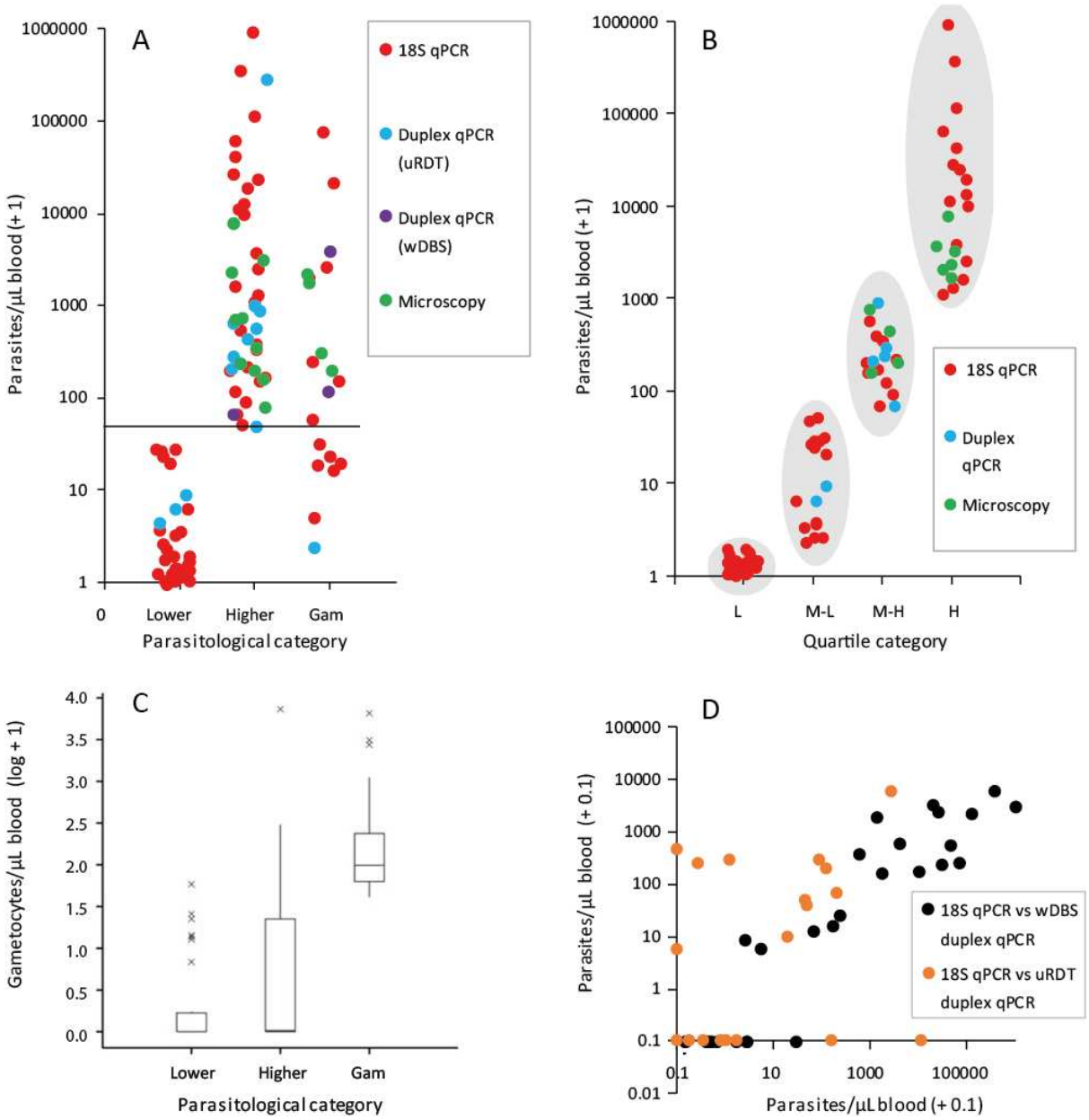
Categories	Description	Infection parameters	Assay	Result	*n*[a]
***P. falciparum* no gametocytes**	*Plasmodium* parasites present but all negative for *P. falciparum* gametocytes by microscopy and QT-NASBA.	*P. falciparum*	18S qPCR	41.04 median p/µL[b]	10
		P. falciparum gams[c]	QT-NASBA	0	
		P. malariae	nPCR[d]	Neg[e]	
		P. ovale	nPCR	Neg	
***P. falciparum* sub-microscopic gametocytes**	*Plasmodium* parasites present, no microscopic gametocytes.	*P. falciparum*	18S qPCR	5208.2 median p/µL	14
		P. falciparum gams	QT-NASBA	14.62 median p/µL	
		P. malariae	nPCR	Neg (*1/14 not tested*)[f]	
		P. ovale	nPCR	Neg (*1/14 not tested*)	
***P. falciparum* high-density gametocytes**	*Plasmodium* parasites present, high density gametocytes (12/13 with microscopic gametocytes).	*P. falciparum*	18S qPCR	96.68 median p/µL	13
		P. falciparum gams	QT-NASBA	660.36 median p/µL	
		P. malariae	nPCR	Neg (*2/13 not tested*)	
		P. ovale	nPCR	Neg (*2/13 not tested*)	
Parasite-free	Negative by all diagnostic measures, including one molecular assay, but allowing RDT positivity[g]	*P. falciparum*	18S qPCR	0	15
		P. falciparum gams	QT-NASBA	0	
		P. malariae	nPCR	Neg (*2/15 not tested*)	
		P. ovale	nPCR	Neg (*2/15 not tested*)	

[a]N=number of odor samples
[b]P/µ=parasites/µL blood
[c]Gams=gametocytes
[d]nPCR=nested PCR
[e]Neg = negative
[f]It was not possible to test all samples for *P. malariae* or *P. ovale* due to insufficient DNA template
[g]RDT positivity (9/15 samples) was allowed on the basis that these tests can remain positive for some time following curative treatment, due to circulating HRP-2 protein

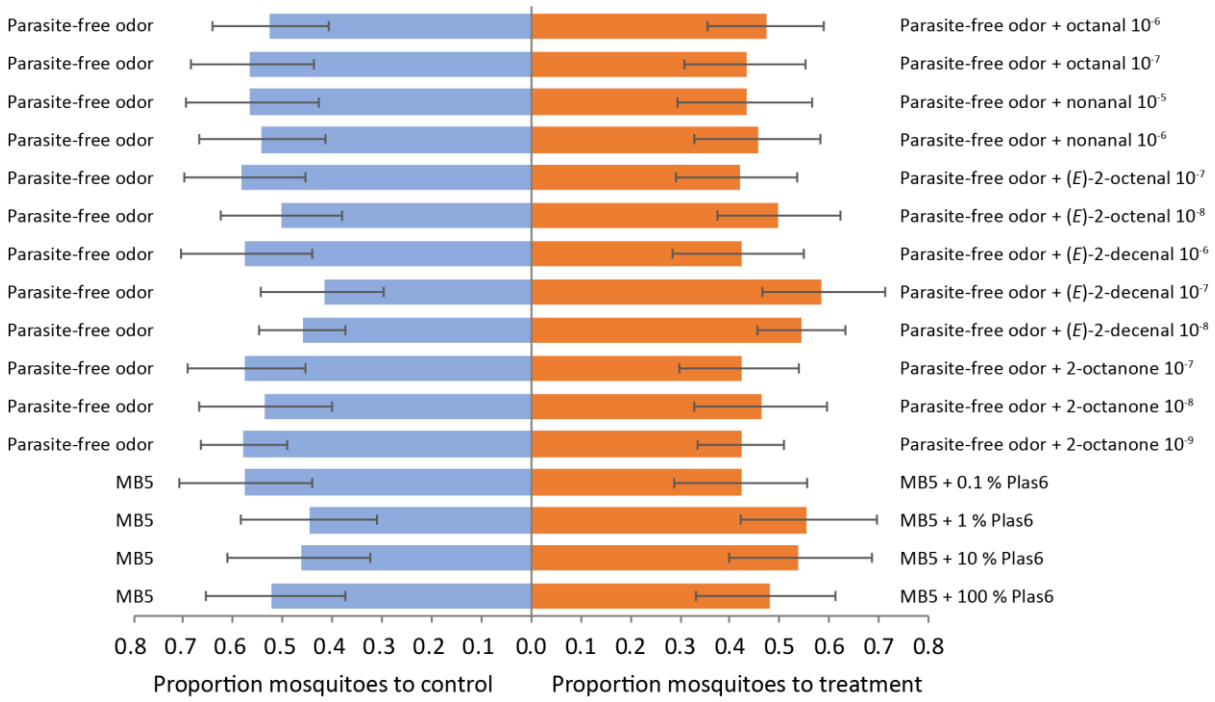
S3. Analytes (compounds eluting during gas chromatography, sometimes at the same time) within odor samples that were found to induce antennal response in *Anopheles coluzzii*. The identification of analytes in bold font was subsequently confirmed by co-injection with an authentic standard.

RI[(a)]	TENTATIVE IDENTIFICATION
716	Methylcyclohexane
750	Unknown
781	Dimethyl sulfide
795	Solvent
801	Octane
853	1-Hexanol (unsure identification), dimethyl sulfone
880	**Heptanal**
889	Solvent
933	Benzaldehyde
958	Phenol/1-octen-3-one
982	**Octanal**
1014	2-Ethylhexanol or 2-octene
1056	Shoulder para cresol (4-methylphenol) and 3-octen-2-ol, peak octen-1-ol
1084	**Nonanal**
1118	Unknown
1139	2-Ethylbenzaldehyde
1150	4-Ethylbenzaldehyde
1186	Decanal
1199	Dodecane
1239	Ethylacetonphenone
1389	Unknown
1428	Geranylacetone

[(a)]Analytes identified by retention index (RI) were selected if eliciting >3 responses in one of the treatment groups (*Plasmodium* infection, no gametocytes, *n*=6; *Plasmodium* infections, sub-microscopic *P. falciparum* gametocytes, *n*=7; high-density *P. falciparum* gametocytes, *n*=7; parasite-free individuals, *n*=7; control, *n*=7)



S4. (**A**) 'Total density' parasitological categorization, showing actual *Plasmodium* parasite densities p/µL) per group (Lower, total <50 p/µL blood; Higher, total >50 p/µL blood; Gam, gametocyte carriers by microscopy). Here total parasites (all stages) are shown. Note, of Gametocyte category samples, 65 % harbored total parasite densities of >50 p/µL (higher category). Colors represent the diagnostic technique used to inform categorization. (**B**) 'Quartile' parasitological categorization, showing actual parasite densities (p/µL) per group. Here all 'higher density' and 'lower density' samples from the 'total density' categories were re-classified: L=low, mean/median parasite density 0.38/0.3, *n*=21; M-L=medium-low, mean/median parasite density 16.77/8.3, *n*=17; M-H=medium-high, mean/median parasite density 296.60/214.18, *n*=19; H=high, mean/median parasite density 102669.46/13304.54, *n*=23. Colors represent the diagnostic technique used to inform categorization. (**C**) Boxplots of median gametocyte densities per µL blood (on log-scale, boxes representing the interquartile range, median being the bottom of the box for 'Lower' (QT-NASBA mean/median parasite density 4.82/0, *n=31*) and 'Higher' (QT-NASBA mean/median parasite density 234.51/0.030, *n=38*) or the line in the box for 'Gametocytes' (QT-NASBA mean/median parasite density 3996.84/351.58, *n=16*, microscopy 758.40/100, *n=20)*), in 'total density' categories. (**D**) Correlation in parasite density as measured by 18S qPCR vs. PgMET qPCR, the latter amplifying from either a Whatman filter paper dried blood spot template (wDBS), or a used rapid diagnostic test template (uRDT) for when wDBS was unavailable. Correlations shown only for individuals in the 'Lower' and 'Higher' density group (**A**).



S5. ***Anopheles coluzzii*** **responses in a dual-port olfactometer to infection-associated compounds (IAC) alone and in a blend, 'Plas 6' (orange bars), relative to background odor alone (blue bars).** IAC at two concentrations (g/mL) were presented with, and tested against, odor (socks) from parasite-free study participants (5-12 year-old Kenyan children), over 8/9 replicates. Heptanal was presented with, and tested against, the synthetic lure MB5 (ammonia, L-(+)-lactic acid, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine), at three concentrations over 11/12 replicates. Plas 6 (heptanal, octanal, nonanal, (*E*)-2-octenal, (*E*)-2-decenal and 2-octanone) was also presented with, and tested against, the synthetic lure MB5, at four concentrations and over 10/11 replicates. Each replicate tested 30 mosquitoes. Predicted mean proportions and 95 % confidence intervals (CI) are presented, from six generalized linear models (GLMs) assuming a Binomial distribution and using a logit link function (See Table S9 for details of the GLMs). No treatments were preferable to controls, with all 95 % CI including 0.5.

S6. Significance (F-tests) of covariates and factors from linear mixed modelling (REML) for infection-associated compound production predicted by 'total density', 'quartile', or 'positive vs. negative' categories.

Compound	Co-v/Factors	n.d.f. [a]	d.d.f. [a]	F. statistic	*P* value	Categories with significant pairwise differences
'Total density' categories						
Heptanal	P/C: total density[b]	3	179.6	2.24	0.085	- Higher vs. negative - Lower, higher and gametocyte vs. CNL(A)/(B) - Negative vs. CNL(B) only
	Sample type[c]	2	8.1	31.21	<0.001	
Octanal	P/C: total density	3	174.1	2.88	0.038	- Higher vs. lower - Lower, higher and gametocyte vs. CNL(A)/(B) - Negative vs. CNL(B) only
	Sample type	2	7.8	47.85	<0.001	
	Age[d]	1	97.1	10.40	0.002	
	Age^2	1	94.8	5.59	0.020	
	Sex[e]	1	87.4	9.95	0.002	
Nonanal	P/C: total density	3	176.2	3.13	0.027	- Higher vs. lower - Negative, higher and gametocyte vs. CNL(A)/(B) - Lower vs. CNL(B) only
	Sample type	2	7.7	44.86	<0.001	
	Hb[f]	1	181.9	4.54	0.034	
	Hb^2	1	181.3	6.92	0.009	
(*E*)-2-Octenal	P/C: total density	3	160.4	1.80	0.150	Lower, higher and gametocyte vs. CNL(A)/(B)
	Sample type	2	8.4	8.53	0.010	
	Hb	1	180.3	6.42	0.012	
	Round[g]	3	125	3.75	0.013	
(*E*)-2-Decenal	P/C: total density	3	172.7	1.53	0.209	Lower, higher and gametocyte vs. CNL(A)/(B)
	Sample type	2	9.7	11.68	0.003	
	Age	1	78.7	13.29	<0.001	
2-Octanone	P/C: total density	3	176.4	5.41	0.001	- Gametocyte vs. negative, lower, higher, CNL(A)/(B) - Lower and higher vs. CNL(A) and (B)
'Quartiles' categories						
Heptanal	P/C: quartiles	5	178	1.19	0.317	No important pairwise differences
	Sample type	2	8.1	31.65	<0.001	
Octanal	P/C: quartiles	5	171.6	1.26	0.284	No important pairwise differences
	Sample type	2	7.8	47.49	<0.001	
	Age	1	100	8.69	0.004	
	Age^2	1	97.9	4.67	0.033	
	Sex	1	89.5	9.86	0.002	
Nonanal	P/C: quartiles	5	176.4	1.82	0.111	Low vs. High
	Sample type	2	7.8	42.69	<0.001	
'Positive vs. negative' categories[h]						
(*E*)-2-Octenal	P/C: positive vs. negative	1	173.1	4.28	0.040	Positive vs. CNL(A)/(B)
	Sample type	2	8.40	8.54	0.010	
	Hb	1	182.1	6.47	0.012	
	Round	3	123.9	4.01	0.009	
(*E*)-2-Decenal	P/C: positive vs. negative	1	179.9	4.20	0.042	Positive vs. negative, CNL(A)/(B)
	Sample type	2	9.9	11.79	0.002	
	Age	1	79.2	13.37	<0.001	

[a] n.d.f.=numerator degrees of freedom for F-test, d.d.f.=denominator degrees of freedom for F-test
[b] P/C=parasitological category
[c] Sample type=human odor sample or control (solvent [CNL(A)] or empty bag [CNL(B)])
[d] Age=age (years) of child from whom odor sample was taken
[e] Sex=sex of child from whom odor sample was taken
[f] Hb (g/dL)=Hb level of child from whom odor sample was taken
[g] Round = round of sampling (1-3)
[h] Grouping all *Plasmodium*-positive individuals together (positive, *n*=101; negative, *n*=16)

S7. Lower triangular matrices of pairwise comparisons of means of interest (Fig. 3) between groups for infection-associated compounds, according to parasitological categorization ('total density', 'quartiles' and 'postive vs. negative') used in linear mixed models (REML). Standard errors of differences (SED) between groups are shown.

HEPTANAL (TOTAL DENSITY CATEGORIES)						
Category	**SED**					
Higher density						
Gametocytes	0.129					
Lower density	0.108	0.139				
Negative	0.140 +	0.164	0.151			
CNL(A)	0.346 +	0.356 +	0.350 +	0.361		
CNL(B)	0.095 +	0.128 +	0.108 +	0.137 +	0.345	
df = 179, av. SED=0.2	Higher density	Gametocytes	Lower density	Negative	CNL(A)	CNL(B)

HEPTANAL (QUARTILE CATEGORIES)[(a)]							
Category	**SED**						
1							
2	0.158						
3	0.154	0.160					
4	0.145	0.155	0.150				
CNL(B)	0.125 +	0.135 +	0.128 +	0.121 +			
CNL(A)	0.351 +	0.356 +	0.353 +	0.350 +	0.341		
Gametocytes	0.153	0.161	0.157	0.149	0.129 +	0.352 +	
Negative	0.163	0.171	0.164	0.160	0.138 +	0.357	0.165
df=178, av. SED=0.20	1	2	3	4	CNL(B)	CNL(A)	Gametocytes

OCTANAL (TOTAL DENSITY CATEGORIES)						
Category	**SED**					
Higher density						
Gametocytes	0.589					
Lower density	0.489 +	0.631				
Negative	0.634	0.745	0.686			
CNL(A)	1.683 +	1.725 +	1.699 +	1.743		
CNL(B)	0.434 +	0.585 +	0.492 +	0.624 +	1.678	
df = 174, av. SED=0.96	Higher density	Gametocytes	Lower density	Negative	CNL(A)	

OCTANAL (QUARTILE CATEGORIES) [(a)]							
Category	**SED**						
1							
2	0.732						
3	0.705	0.736					
4	0.662	0.716	0.686				
CNL(B)	0.577 +	0.624 +	0.590 +	0.558 +			
CNL(A)	1.708 +	1.731 +	1.717 +	1.703 +	1.664		
Gametocytes	0.702	0.737	0.724	0.683	0.592 +	1.714 +	
Negative	0.749	0.792	0.745	0.734	0.632 +	1.732	0.756
df = 171, av. SED=0.94	1	2	3	4	CNL(B)	CNL(A)	Gametocytes

NONANAL (TOTAL DENSITY CATEGORIES)					
Category	**SED**				
Higher density					
Gametocytes	1.802				
Lower density	1.508 +	1.943			
Negative	1.974	2.312	2.097		
CNL(A)	5.804 +	5.921 +	5.844	5.972 +	
CNL(B)	1.321 +	1.783 +	1.499 +	1.924 +	5.789

df = 176, av. SED =3.7	Higher density	Gametocytes	Lower density	Negative	CNL(A)
NONANAL (QUARTILE CATEGORIES) [(a)]					
Category	**SED**				
1					
2	2.247				
3	2.183	2.258			
4	2.057 +	2.198	2.123		
CNL(B)	1.778 +	1.912 +	1.823 +	1.720 +	
CNL(A)	5.657 +	5.715 +	5.682 +	5.642 +	5.530
Gametocytes	2.169	2.282	2.236	2.115	1.830 +
Negative	2.316	2.429	2.323	2.274 +	1.958 +
df =176, av. SED=3.01	1	2	3	4	CNL(B)

5.672 +	
5.727	2.342
CNL(A)	Gametocytes

(*E*)-2-OCTENAL (TOTAL DENSITY CATEGORIES)					
Category	**SED**				
Higher density					
Gametocytes	0.177				
Lower density	0.153	0.177			
Negative	0.189	0.214	0.188		
CNL(A)	0.320 +	0.333 +	0.318 +	0.336	
CNL(B)	0.123 +	0.184 +	0.162 +	0.190	0.320
df = 160, av. SED=0.23	Higher density	Gametocytes	Lower density	Negative	CNL(A)

(*E*)-2-OCTENAL (POSTIVE VS. NEGATIVE CATEGORIES)			
Category	**SED**		
Positive			
Negative	0.182		
CNL(A)	0.320 +	0.340	
CNL(B)	0.107 +	0.189	0.324
df = 179, av. SED=0.73	Positive	Negative	CNL(A)

(*E*)-2-DECENAL (TOTAL DENSITY CATEGORIES)					
Category	**SED**				
Higher density					
Gametocytes	0.534				
Lower density	0.441	0.570			
Negative	0.571	0.673	0.616		
CNL(A)	0.940 +	1.002 +	0.962 +	1.025	
CNL(B)	0.395 +	0.531 +	0.445 +	0.565	0.932
df = 172, av. SED=0.68	Higher density	Gametocytes	Lower density	Negative	CNL(A)

(*E*)-2-DECENAL (POSTIVE VS. NEGATIVE CATEGORIES)			
Category	**SED**		
Positive			
Negative	0.549 +		
CNL(A)	0.927 +	1.038	
CNL(B)	0.338 +	0.573	0.940
df = 179, av. SED=0.73	Positive	Negative	CNL(A)

2-OCTANONE (TOTAL DENSITY CATEGORIES)					
Category	**SED**				
Higher density					
Gametocytes	0.013 +				
Lower density	0.011	0.014 +			
Negative	0.014	0.016 +	0.014		
CNL(A)	0.019 +	0.021 +	0.019 +	0.021	
CNL(B)	0.009 +	0.012 +	0.010 +	0.0128	0.019

df = 176, av. SED=0.01	Higher density	Gametocytes	Lower density	Negative	CNL(A)

+ Significance (P<0.05) for comparisons made using the least significant difference (LSD, 5 %) values (= SED multiplied by the 2.5 % t-value)

[a]For 'Quartile' categories, 1=low, 2=medium-low, 3=medium-high, 4=high

S8. Experimental design for 100 % blends of infection-associated compounds (IAC), Plas5 and Plas6.

IAC	**Plas5 %**	**Plas5 proportion**[d]	**Plas6 %**	**Plas6 proportion**[d]
Nonanal[a]	16.36	1.00	16.36	1.00
Octanal[a]	5.30	0.32	5.30	0.32
Heptanal[a]	0.98	0.06	0.98	0.06
(*E*)-2-octenal[b]	0.64	0.04	0.64	0.04
(*E*)-2-decenal[b]	2.11	0.13	2.11	0.13
2-octanone[c]			0.09	0.01

[a]The predicted proportion of each compound found in the 'higher' density group ('total density' categorization, REML) was used to generate values for octanal, nonanal and heptanal as the production of these compounds was upregulated in these groups

[b]The predicted proportion found in the 'positive' group ('positive vs. negative' categorization, REML) was used

[c]The predicted proportion was taken from the 'gametocyte' group, 'total density' categorization

[d]Ratios were derived from these proportions, then all were normalized to the actual mean amount of nonanal found in the 'higher' density category (476 ng) in 100 min

S9. Significance (F-tests) of covariates and factors included in generalized linear models, assuming a Binomial distribution and using a logit link function, testing the preference of *Anopheles coluzzii* mosquitoes in a dual-port olfactometer for infection-associated compounds against a background of parasite-free odor or heptanal against the background of the synthetic lure MB5 (IAC alone), or for Plas blends against a background of the synthetic lure MB5 (IAC Plas blends), in dual-port olfactometer assays. N/S = non-significant.

IAC alone[a]	***P* value**				
Factors/variates included in models	**Set 1**	**Set 2**	**(*E*)-2-decenal low**[b]	**2-octanone low**[b]	**heptanal**[c]
Treatment	0.505	0.021	N/S	N/S	0.92
Treatment side[d]	<0.001	<0.001	<0.001	N/S	N/S
Flight chamber[e]	N/S	N/S	0.342	N/S	0.036
Treatment side * Flight chamber	N/S	N/S	0.017	N/S	N/S
Wind speed[f]	N/S	N/S	N/S	N/S	0.027
IAC Plas blends[g]	***P* value**				
Factors/variates included in model					
Treatment (blend)	0.217				
Concentration	0.610				
Treatment (blend)*Concentration	0.486				
Treatment side	<0.001				

[a] IAC alone: two sets of three compounds each (set 1, nonanal, (*E*)-2-decenal, 2-octanone; set 2, octanal, heptanal, (*E*)-2-octenal) were run as separate experiments and analyzed in two separate models. Treatment = IAC at each of two concentrations (nonanal, $10^{-6}/10^{-5}$; 2-octanone, $10^{-8}/10^{-7}$; (*E*)-2-decenal, $10^{-7}/10^{-6}$; octanal, $10^{-7}/10^{-6}$; heptanal, $10^{-8}/10^{-7}$; (*E*)-2-octenal, $10^{-8}/10^{-7}$). Each combination of compound and concentration was thus considered a separate level in these models

[b] Additionally, (*E*)-2-decenal and 2-octanone were tested at a tenfold lower concentration (10^{-8} and 10^{-9} respectively) at later dates, and results were analyzed using two separate models

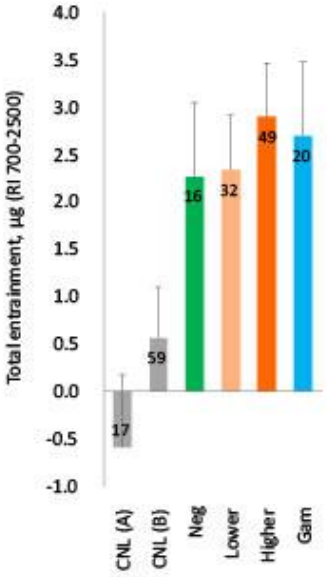
[c] Additionally, heptanal was tested against a background of MB5 in a separate experiment.

[d] Treatment side refers to the olfactometer port used for treatment odor. A significant effect was observed in some experiments where mosquito preference for the left or right port in the triple dual-port olfactometer was biased due to unknown causes and irrespective of the odor source offered. Importantly, the experiments were performed in a balanced way, with the same treatment tested equally often in, and randomized to, the left or right port to minimize the impact of this side-bias on the preference tests.

[e] Flight chamber refers to the flight chamber of the triple dual-port olfactometer.

[f] Wind speed refers to the wind speed measured in front of the port used for treatment odor.

[g] For IAC Plas blends: treatment and concentration were separate factors, and their interaction (indicated by *) was tested.



Parasitological Grouping	Co-v/Factors	n.d.f. [a]	d.d.f. [a]	F. statistic	*P* value
'Total density' categories	P/C: total density [b]	3	179.8	0.3	0.828
	Sample type [c]	2	161.5	17.72	<0.001
	Day of the year [d]	1	147.1	41.92	<0.001
	Day of the year²	1	148.8	4.46	0.036
	Day of the year * sample type	1	136.4	6.1	0.015
'Positive vs. negative' categories [e]	P/C: positive vs. negative [b]	1	185.3	0.02	0.895
	Sample type [c]	2	163.5	17.71	<0.001
	Day of the year [d]	1	146.8	41.63	<0.001
	Day of the year²	1	148.3	4.51	0.035
	Day of the year * sample type	1	138.7	6.22	0.014

[a] n.d.f.=numerator degrees of freedom for F-test, d.d.f.=denominator degrees of freedom for F-test
[b] P/C=parasitological category
[c] Sample type=human odour sample or control (solvent [CNL(A)] or empty bag [CNL(B)])
[d] Number of days since 1st January 2014
[e] Grouping all *Plasmodium*-positive individuals together (positive, *n*=101; negative, *n*=16)

S10. Total amount of all compounds produced (with retention time [RI] between 700 and 2500) by individuals of differing parasitological status. (**A**) Predicted means (+SE) given by linear mixed modelling (REML). There were no significant differences in total amount produced between any of the human groups ('Neg'=negative, 'lower' and 'higher' refer to parasite densities of lesser or greater than 50 p/µL, 'Gam'=microscopic gametocytes, CNL(A)=solvent control, CNL(B)=empty bag control), tested by Least Significant Difference ($P<0.05$). Sample size in bar ends. (**B**) Significance of covariates and factors from linear mixed modelling (REML) for total amount of sample. Both 'total density' and 'positive vs negative' categories were attempted.